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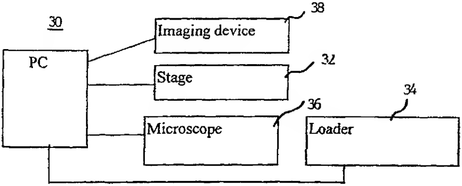

(57) Abstract

A method of and system for detecting and analyzing rare cells in a mixed population of cells carried by a carrier (10). The method is effected by (a) using an automatic or semi-automatic optical scanning system (30) for morphologically identifying the rare cells on the carrier (10), to thereby obtain identified rare cells of knows locations; and (b) chemically, biochemically and/or genetically analyzing the identified rare cells.

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SYSTEM AND METHOD FOR IDENTIFYING AND ANALYZING RARE CELL TYPES IN A MIXED POPULATION OF CELLS

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to a system and method for identifying and analyzing rare cell types in a mixed population of cells. In particular, the present invention relates to an automatic or semi-automatic system and method for morphologically identifying and morphologically and chemically, biochemically and/or genetically analyzing fetal cells, which are rare cells, in maternal blood derived cells, which form a mixed population of cells. Most particularly, the present invention relates to a system and method for automatic or semi-automatic prenatal diagnosis of chromosomal abnormalities in maternal blood derived fetal cells. The present invention also relates to an automatic or semi-automatic system and method for morphologically identifying and morphologically and chemically, biochemically and/or genetically analyzing rare tumor cells in blood derived cells of a cancer patient.

The system and method according to the present invention requires, in principle, no biological enrichment processes prior to implementation and can identify and analyze the rare cells, e.g., fetal or cancer cells, via a fully- or semi-automated screening system, which employs a combination of image processing techniques.

Furthermore, according to preferred embodiments, the system and method of to the present invention synergistically combines between rare cell detection, which is based on morphological features characterizing the rare cells, and rare cell chemically, biochemically and/or genetically analysis which is based on characterization of cellular markers, such as chromosomal markers and/or antigen markers, to thereby synergistically further validate the results.

Thus, the system and method of the present invention are particularly applicable to detection and analysis of fetal cells in maternal derived blood.

In addition, the system and method of the present invention are also applicable to the detection of tumor cells in cancer patient derived blood, and as such can be utilized to monitor and detect what in commonly referred to as Minimal Residual Disease (MRD).

The identification and subsequent analysis of rare cells types found in a population of other cells types is of great importance to medical science.

For example, identification and genetic analysis of fetal cells in maternal blood is of importance in prenatal diagnosis of fetal disorders, fetal gender determination and the like.

The identification and analysis of rare tumor cells using chemically, biochemically and/or genetically can be utilized to detect Minimal Residual Disease (MRD).

At present, there are no automated systems available which enable the morphological identification and subsequent combined morphological and chemical, biochemical and/or genetic analysis of individual rare cells. As such, identification and chemical, biochemical or genetic analysis of rare cell types is currently practiced on a very limited basis and is typically effected by manual detection and counting of rare cells and subsequent analysis of another independent, group of such cells taken from the same patient.

Although the prior art describes systems which apparently can be utilized to enrich and/or chemically, biochemically and/or genetically analyze rare cell types, such systems are typically dedicated in nature, providing enrichment and/or genetic analysis of only one or several specific cell types.

For example, WO 94/02646 describes a method for enriching and interrogating rare fetal cells. This method employs cell-specific antibodies to effect rare cell enrichment. Following enrichment, the rare cells can be genetically, chemically or biochemically interrogated via one or more prior

art techniques.

The limitations inherent to the above described method and additional prior art methods prevent them from being implemented on a commercial scale since such methods are dedicated to one or several cell types, are cumbersome and time consuming to employ and oftentimes provide unreliable results which can render such methods useless.

For example, in the case of fetal cells in maternal blood, the lack of efficient ability to identify rare cells at a concentration less than 1:100,000 sets a barrier for a safe prenatal test. In another example, in the case of MRD, the practical inability to identify and co-analyze rare cancer cells for morphological and chemical, biochemical and/or genetic .characteristics limits the accuracy of the test.

Fetal cells in maternal blood

The existence of fetal cells in maternal blood circulation presents an opportunity for a risk free prenatal diagnosis test which may be performed at an early stage of gestation. There is evidence suggesting that such cells may be found in the maternal blood system as early as six weeks into gestation. These cells contain the genetic information of the fetus and therefore, if isolated, allow examination of fetal chromosomes for abnormalities, such as Downs Syndrome (chromosome 21 trisomy). Many researchers have attempted at developing methods for isolation of maternal blood fetal cells and to thereby develop a minimal risk test for detection of chromosomal abnormalities thereof. This task is, however, extremely challenging, because of the rarity of fetal cells in the maternal blood and the difficulty in distinguishing between fetal cells and maternal blood cells.

The presence of fetal cells circulating in maternal blood has been known for more than a century. In 1893, Schmorl [1] has demonstrated the presence of trophoblasts in the maternal blood system. In 1969 Walknowska et al. [2] showed the existence of the male specific Y chromosome in blood samples taken from 21 pregnant women, 19 of whom

subsequently delivered male infants; the other two had female infants. These findings were later validated by other investigators, who reported the presence of cells with Y (male) chromatin in the circulation of women pregnant with male fetuses.

These findings urged many researchers to try and isolate these fetal cells for use as a tool to examine the fetus for chromosomal abnormalities. Three types of fetal cells can be used for analysis: trophoblasts, lymphocytes and nucleated erythrocytes.

Trophoblasts: Trophoblasts are extremely rare (about 1/1,000,000 cells in erythrocyte depleted maternal blood sample), multinucleated (30-50 nuclei, each of about 10 μm in diameter) and exceptionally large fetal cells (100-200 μm in diameter) present in maternal blood samples. Trophoblasts are not ideal for prenatal diagnosis for several reasons. First, there is a very small number of trophoblasts present in maternal blood during the first trimester of gestation. Second, trophoblasts that are released into the maternal blood quickly become trapped in the lungs and as such these cell types rarely remain in peripheral circulation. Being multinucleated, trophoblasts do not share the same chromosome complement as other fetal cells and are therefore bad candidates for chromosomal analysis.

Leukocytes: Fetal leukocytes are an attractive potential source of cells for prenatal diagnosis. A potential problem is that in some cases such cells can persist in the maternal circulation for many years after pregnancy. Bianchi et al. found signs of male fetal cells containing the Y chromosome), in six of eight non-pregnant women who gave birth to a male child and were 6 months to 27 years post partum [3]. It is evident in this respect that the presence of leukocytes of a former fetus may interfere with the analysis of leukocytes of a present fetus.

Nucleated Erythrocytes: Fetal nucleated erythrocytes (Nucleated Red Blood Cells - NRBC), appear to be most suited for prenatal diagnosis of maternal blood circulating fetal cells for several reasons. First, maternal

nucleated erythrocytes are rare in the adult circulation (except under clinical circumstances), but are common in the fetus, especially during early gestation stages. Second, fetal nucleated erythrocytes express several unique antigens, such as the transferring receptor. Third, fetal nucleated erythrocytes produce unique fetal hemoglobin chains such as zeta (ζ) and gamma (γ) hemoglobin [4], which have the potential to be used as cellular markers to identify these fetal cells. Finally, erythrocytes are known to have a short life span and are unlikely to persist from one pregnancy to the another [5].

Frequency of fetal cells in maternal blood: Isolation of fetal cells from maternal blood has been particularly challenging because of their rarity. Estimates of the occurrence of fetal cells in maternal blood vary [6], ranging from 1 in 10^5 to 1 in 10^9. Hamada et al. used Fluorescence In Situ Hybridization (FISH) on unsorted maternal blood to find evidence of cells bearing the Y chromosome. The number of such cells increased as gestation progressed from less than 1 in 10^5 in the first trimester to 1 in 10^4 at term [7]. Another intriguing question concerns the timing of the entrance of fetal cells into the maternal circulation. Available data demonstrates that detection of fetal DNA derived sequences is possible even in the first trimester. In a study of two women carrying male fetuses conceived by *in vitro* fertilization, fetal DNA was detected in maternal blood as early as 33 and 40 days into gestation [8]. Figure 1 shows the average number of nucleated erythrocytes in 7 ml of maternal blood as reported by one research group [9]. Table 1 below summarizes some experiments for sex determination from fetal cells or DNA in maternal blood. The results show that such identification may be performed as early as 6 weeks of gestation.

Table 1

Prenatal sex determination from fetal cells or DNA in maternal blood

First Author (Year) [Ref]	r of s	Weeks of gestation	Overall accuracy
Bianchi (1990) [11]	19	12-17	89%
Bianchi (1991) [12]	7	11-20	100% at 11-13 weeks 25% at 13-20 weeks
Bianchi (1993) [13]	47	8-19	65% with one technique; 100% with another technique
Hamada (1993) [7]	17	7-19	100% at 15-19 weeks by FISH 45% at 7-14 weeks by FISH
Liou (1994) [14]	19	Weekly 6-12 weeks	84% by 10 weeks 100% by 12 weeks
Lo (1990) [15]	15	6-15	80%
Lo (1993) [16]	30	6-18	70%
Von Koskull (1995) [17]	13	6-19	85%
Wechtel (1996) [18]	40	10-19	unsorted 64% sorted 94%
Zheng (1993) [19]	6	13-17	83%

Currently there is no commercially available test which exploits a maternally derived blood sample for identifying chromosomal abnormalities in fetuses. A great deal of effort is invested into developing such a test. Such efforts are mainly directed at elimination of maternal cells from the blood sample by various processes, in order to enrich and concentrate the fetal cells present in the sample. Such methods are therefore referred in the relevant literature as "enrichment processes". To date there are four enrichment processes that are common: (i) density gradient; (ii) Fluorescence Activated Cell Sorting (FACS); (iii) Magnetic Activated Cell Sorting (MACS); and (iv) charge flow separation (and/or electrophoresis).

Thus, due to the extreme rarity of fetal cells in maternal blood, a number of specialized techniques to enrich and/or isolate the fetal cell fraction or the fetal genetic material from maternal blood have been designed.

Although limited separation can be achieved using such methods,

these methods are typically not sensitive enough to effect the isolation of a fetal cell fraction usable for highly reliable genetic testing, e.g., substantially zero tolerance. In addition, the currently used enrichment methods such as gradient centrifugation all result in substantial cell loss, thereby reducing the number of fetal cells available for subsequent analysis or for use with subsequent cell sorting or enrichment techniques.

A more sensitive approach that has been utilized in an attempt to isolate fetal cells from a maternal blood sample utilizes labeled antibodies specific for a particular fetal cell type. Antibody labeled cells can then be isolated by a variety of methods which depend on the recognition of the antibody label. For example, fetal cell specific antibodies can be used to label fetal cells in order to facilitate separation of these cells from maternal cellular components by flow cytometry (Herzenberg, L. A., et al., Proc. Natl. Acad. Sci. USA 76, 1453-1455 (1979); Iverson, G. M., et al., Prenatal Diagnosis 1, 61-73 (1981); Bianchi, D. W., et al., Prenatal Diagnosis 11, 523-528 (1991) which can utilize fluorescent activation cell sorting (FACS, Herzenberg et al., 1979, Proc. Natl. Aca. Sci. USA 76:1453), magnetic-activated cell sorting (MACS, Ganshirt-Ahlert et al., 1992, Am. J. Obstet. Gynecol. 166:1350) or a combination of both procedures (Ganshirt-Ahlert et al., 1992, Am. J. Hum. Genet. 51:A48). In addition, a combination of gradient centrifugation and flow cytometry methods can also be used to increase the isolation or sorting efficiency.

Although these non-invasive enrichment methods provide an alternative to the currently used invasive techniques, limitations inherent to their design limits such methods from being widely practiced.

For example, a major limitation inherent to the flow cytometry techniques arises from the antibodies utilized by such techniques. Such antibodies, although generated cell specific, often cross react with other unwanted cell types which are in far higher concentration in the sample. As a result, such methods are often only sufficient in enriching for fetal cell

types and cannot be used for reliable, zero tolerance, fetal cell isolation.

Several publications describe the isolation of fetal cells via micromanipulation.

Tutschek B. et al., "Isolation of fetal cells from transcervical samples by micromanipulation: Molecular conformation of their fetal origin and diagnosis of "fetal aneuploidy" Prenatal Diagnosis. Vol. 15: 951-960, 1995, teach the isolation of fetal cells from transcervical samples by micromanipulation to thereby reduce a possibility of co-isolation of maternal cells. However, Tutschek B. et al. fail to teach isolation of rare cells from maternal blood samples.

Takabayashi H. et al. "Development of a non-invasive fetal DNA diagnosis from maternal blood" Prenatal Diagnosis, Vol. 15: 74-77, 1995, teach detection and retrieval of Pappenheim stained fetal nucleated

Cueung M. et al. "Prenatal diagnosis of sickle cell anemia and thalassemia by analysis of fetal cells in maternal blood" Nature Genetics, Vol. 14: 264-268, 1996, teach the scraping of fetal nucleated erythrocytes stained with an anti-fetal globin antibody from microscopic slides.

It will, however, be appreciated that cell identification, prior to micromanipulation is performed manually, which task is both laborious and time consuming.

All of these methods, also referred to herein as "biological processes", are designed to discard all data other than the specific character of the cell which is to be enriched or isolated. These methods suffer from one or more of the following inherent limitations:

Poor end result - Except for micromanipulation, each of the methods described above discard cells of maternal origin, resulting in increase in fetal cell concentration from about $1:10^9$ to about $1:10^5$. These results are not sufficient for a commercially viable test.

Loss of cells - In the above mentioned enrichment techniques,

micromanipulation in particular, there is a risk that the important cells, namely the cells of fetal origin, shall be lost during the enrichment process. Indeed, there are reports demonstrating that as many as 30 % of the fetal cells may be lost in the enrichment process [10].

For these reasons, at present, no system or method exist which enable fetal cell detection combined with subsequent chemical, biochemical and/or genetic analysis of the detected fetal cells.

The current research trend in identification and isolation of fetal cells is based on the concept that there is a single or a small number of parameters that distinguish fetal cells from maternal cells. Thus, each of the enrichment methods described above employs a single fetal cell parameter in order to separate such cells from maternal cells. In the case of density gradient, for example, it is the weight of the cells, in the case of FACS and MACS it is a cellular antigen. The problem is, however, that there is presently no single effective differentiation, and it seems that a better way to distinguish between one group of cells to another will be by employing a set of different parameters, each of which contributes to an identification decision making in accordance with its weight.

Thus, the detection and analysis method of the present invention employs a set of distinguishable parameters which when used in combination can differentiate and thus enable the detection of a variety of rare cell types in a population of other cell types. The present invention also combines this detection with subsequent chemical, biochemical and/or genetic analysis of the detected cells.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a sample carrier for mounting over a microscope stage and for placement in a swinging bucket centrifuge, the sample carrier comprising a surface area greater than about 3,500 squared millimeters, the surface area being formed

with at least 50 sample wells.

According to another aspect of the present invention there is provided a dispenser comprising a container being formed with a plurality of holes, the dispenser being adapted to match to a sample carrier including a surface area greater than about 3,500 squared millimeters, the surface area being formed with at least 50 sample wells, such that when the dispenser is mounted over the carrier, the plurality of holes of the container face the plurality of wells of the carrier, so as to facilitate dispensing of a sample through the holes into the wells.

According to yet another aspect of the present invention there is provided a sample preparation assembly comprising (a) a dispenser including a container being formed with a plurality of holes; (b) a sample carrier including a surface area greater than about 3,500 squared millimeters, the surface area being formed with at least 50 sample wells, such that when the dispenser is mounted over the carrier, the plurality of holes of the container face the plurality of wells of the carrier, so as to facilitate dispensing of a sample through the holes into the wells.

According to still another aspect of the present invention there is provided a method of dispensing a sample, the method comprising the steps of (a) providing a preparation assembly including (i) a dispenser including a container being formed with a plurality of holes; and (ii) a sample carrier including a surface area greater than about 3,500 squared millimeters, the surface area being formed with at least 50 sample wells; (b) mounting the dispenser over the carrier such that the plurality of holes of the container face the plurality of wells of the carrier, so as to facilitate dispensing of the sample through the holes into the wells; and (c) dispensing the sample through the holes into the wells.

According to an additional aspect of the present invention there is provided a method of analyzing a sample, the method comprising the steps of dispensing the sample by (a) providing a preparation assembly including

(i) a dispenser including a container being formed with a plurality of holes; and (ii) a sample carrier including a surface area greater than about 3,500 squared millimeters, the surface area being formed with at least 50 sample wells; (b) mounting the dispenser over the carrier such that the plurality of holes of the container face the plurality of wells of the carrier, so as to facilitate dispensing of the sample through the holes into the wells; and (c) dispensing the sample through the holes into the wells; and analyzing a content of at least one of said wells.

According to further features in preferred embodiments of the invention described below, the sample carrier further comprising at least two feducial markers.

According to still further features in the described preferred embodiments the feducial markers feature a cross hair configuration.

According to still further features in the described preferred embodiments the container includes partitions dividing the container into chambers, whereas each of the chambers is in fluid communication with at least one of the holes.

According to still further features in the described preferred embodiments the holes are positioned at distal ends of protrusions protruding externally to the container.

According to still further features in the described preferred embodiments the step of dispensing the sample through the holes into the wells is effected under centrifugal force.

According to still further features in the described preferred embodiments provisions are taken to ensure that each well includes a monolayer of cells derived from the sample.

According to yet an additional aspect of the present invention there is provided a method of reducing false positive results while identifying rare cells in a mixed population of cells, the method comprising the steps of (a) employing a first algorithm for identifying the rare cells, the first algorithm

identifying the rare cells according to morphological features thereof; and (b) independently employing a second algorithm for identifying the rare cells, the second algorithm identifying the rare cells according to cellular markers characterizing the rare cells; (c) identifying a specific cell of the mixed population of cells as a rare cell only if both the first algorithm and the second algorithm identified the specific cell as rare.

According to yet an additional aspect of the present invention there is provided a method of detecting and analyzing rare cells in a mixed population of cells carried by a carrier, the method comprising the steps of (a) using an automatic or semi-automatic optical scanning system for morphologically identifying the rare cells on the carrier, to thereby obtain identified rare cells of known locations; and (b) chemically, biochemically and/or genetically analyzing the identified rare cells.

According to further features in preferred embodiments of the invention described below, the method further comprising the step of co-displaying a morphological image and an image presenting a chemical, biochemical and/or genetic analysis of the identified rare cells.

According to still an additional aspect of the present invention there is provided a system for identifying rare cells in a mixed population of cells carried by a carrier, the system comprising a scanning unit including an optical magnification device optically communicating with an imaging device and a processing device for analyzing images being acquired by the imaging device, the processing device being for executing an image processing algorithm, the image processing algorithm being for segmenting an image acquired by the imaging device into a first group of pixels associated with cell nuclei, a second group of pixels associated with cell cytoplasms and a third group of pixels associated with background.

According to a further aspect of the present invention there is provided a method of identifying rare cells in a mixed population of cells carried by a carrier, the method comprising the steps of (a) providing a

scanning unit including (i) an optical magnification device optically communicating with an imaging device; and (ii) a processing device being for analyzing images acquired by the imaging device, the processing device being for executing an image processing algorithm; (b) acquiring, via the imaging device, an image of the mixed population of cells; and (c) utilizing the image processing algorithm for segmenting the image of the mixed population of cells acquired by the imaging device into a first group of pixels associated with cell nuclei, a second group of pixels associated with cell cytoplasms and a third group of pixels associated with background to thereby enable identification of the rare cells.

According to further features in preferred embodiments of the invention described below, the first, second and third groups of pixels are segmented according to at least two thresholds calculated by the image processing algorithm, thus, the step of segmenting the image of the mixed population of cells into the first, second and third groups of pixels is effected according to at least two thresholds calculated by the image processing algorithm.

According to still further features in the described preferred embodiments the at least two thresholds are calculated by (i) transforming a color image of the cells into a monochromatic image in which each color channel of each pixel is weighted according to its respective intensity and a monochromatic weighted average intensity is given to each of the pixels; (ii) generating a histogram representing a distribution of monochromatic weighted average intensity distribution among the pixels; and (iii) using the histogram, calculating the at least two thresholds.

According to still further features in the described preferred embodiments segmenting an image acquired by the imaging device into a first group of pixels associated with cell nuclei, a second group of pixels associated with cell cytoplasms and a third group of pixels associated with background is effected by generating at least a nuclei binary image and a

cytoplasm binary image.

According to still further features in the described preferred embodiments the image processing algorithm is further for blob classification, whereas blob classification is effected by, first, identifying connected components in the binary images, thereby identifying blobs in each of the binary images, and thereafter scoring features extracted from the blobs to thereby classify the blobs.

According to still further features in the described preferred embodiments the step of segmenting the image of the mixed population of cells into the first, second and third groups of pixels is effected by blob classification, whereas blob classification is effected by, first, identifying connected components in the binary images, thereby identifying blobs in each of the binary images, and thereafter scoring features extracted from the blobs to thereby classify the blobs.

According to still further features in the described preferred embodiments the features are selected from the group consisting of (i) a size of each of the blobs; (ii) a perimeter of each of the blobs; (iii) a roughness each of the blobs; (iv) a compactness of each of the blobs; (v) an elongation of each of the blobs; (vi) bounding box dimensions; (vii) minimal diameter; (viii) maximal diameter; (ix) mean diameter; (x) convex perimeter; (xi) minimal intensity of a pixel; (xii) maximal intensity of a pixel; (xiii) mean intensity of pixels; (xiv) standard deviation of pixel intensity; (xv) sum of squares of pixels intensities; and (xvi) a number of holes of each of the blobs.

According to still further features in the described preferred embodiments the first group of pixels associated with the cell nuclei is redefined using a blob finding and classification algorithm and a boundary detection algorithm.

According to still further features in the described preferred embodiments the second group of pixels associated with the cell cytoplasms

is redefined using a result of the boundary detection algorithm.

According to still further features in the described preferred embodiments the image processing algorithm is further for matching blobs derived from each of the binary images and thereafter scoring features extracted from matched blobs to thereby classify the blobs.

According to still further features in the described preferred embodiments the features are selected from the group consisting of (i) a size ratio between blobs of matched blobs; (ii) a level of overlap between blobs of matched blobs; (iii) a minimal distance between edges of blobs of matched blobs; and (iv) a maximal distance between edges of blobs of matched blobs.

According to still further features in the described preferred embodiments the image processing algorithm is further for extracting color features of pixels associated with the matched blobs.

According to still further features in the described preferred embodiments the image processing algorithm is further for identifying the rare cells according to mandatory features and sufficient features.

The present invention successfully addresses the shortcomings of the presently known configurations by providing a system and method for identifying and analyzing rare cell types, such as fetal or cancer cells, in a mixed population of cells. The invention synergizes between morphological identification and morphological and chemical, biochemical and/or genetic analysis of such rare cells.

Implementation of the method and system of the present invention involves performing or completing selected tasks or steps manually, automatically, or a combination thereof. Moreover, according to actual instrumentation and equipment of preferred embodiments of the method and system of the present invention, several selected steps could be implemented by hardware or by software on any operating system of any firmware or a combination thereof. For example, as hardware, selected

steps of the invention could be implemented as a chip or a circuit. As software, selected steps of the invention could be implemented as a plurality of software instructions being executed by a computer using any suitable operating system. In any case, selected steps of the method and system of the invention could be described as being performed by a data processor, such as a computing platform for executing a plurality of instructions.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIG. 1 is a histogram showing the average number of nucleated erythrocytes in 7 ml of maternal blood as reported in the prior art;

FIG. 2 is a top view of a sample carrier according to the present invention;

FIG. 3 is a perspective view of a sample dispenser according to the present invention, which can be utilized to dispense a sample onto the sample carrier of the present invention;

FIG. 4 is a black box diagram depicting the various components of the system of the present invention;

FIG. 5 is a flow chart diagram describing the various sample processing steps utilized by the system and method of the present invention;

FIG. 6 is a flow chart diagram describing, in general, the image processing algorithm employed by the system and method of the present invention;

FIG. 7 is a flow chart diagram describing in detail the generation of a histogram according to the image processing algorithm employed by the present invention and the generation of cytoplasm and nuclei binary images thereby.

FIGs. 8-9 illustrate a non-transformed histogram (Figure 8) and a transformed histogram (Figure 9) according to the teachings of the present invention;

FIG. 10 is a graph depicting the curves generated for nucleus, cytoplasmic and associated pixels and the thresholds differentiating there amongst in accordance with the teachings of the present invention;

FIG. 11 is a flow chart diagram detailing the "Blob finding" step implemented by the image processing algorithm of the present invention;

FIG. 12 is a flow chart diagram illustrating, in general, cell classification using the algorithm of the present invention;

FIG. 13 is a flow chart diagram illustrating steps taken for nucleus boundary detection, resulting in nucleus segmentation, implemented by the image processing algorithm of the present invention;

FIG. 14 is a flow chart diagram of cell classification steps implemented by the image processing algorithm of the present invention; and

FIGs. 15a-b depict images of two adjacent cell (Figure 15a) and a single multinucleated cell (Figure 15b) which can be differentiated utilizing the image processing algorithm of the present invention.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of a system and method which can be used for identifying and analyzing rare cell types in a mixed population of cells. Specifically, the present invention can be used for morphologically identifying and morphologically and chemically, biochemically and/or genetically analyzing maternal blood circulating fetal cells or cancer patient circulating tumor cells. In principle, the system and method according to the present invention require no biological elimination and/or enrichment processes prior to implementation and can identify and analyze the rare cells, e.g., fetal or cancer cells, via a fully- or semi-automated screening unit, using solely image processing techniques. The system and method of to the present invention synergistically combines rare cell detection, which is based on morphological features characterizing the rare cells, with subsequent rare cell analysis which is based on characterization of cellular markers, such as chromosomal markers, cellular antigen markers and/or chemical/biochemical markers, to thereby provide results which rely on the synergistic combination of cell morphology and cellular marker analysis. While implementing preferred embodiments of the system and method of the present invention dozens of morphologically related parameters are employed and weighted in order to distinguish the rare cells of interest from the background cell population, to thereby achieve better results.

The principles and operation of a system and method according to the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details of construction and the arrangement of the components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology

employed herein is for the purpose of description and should not be regarded as limiting.

The present invention may be divided into two successive stages: (i) morphological rare cell detection; and (ii) rare cell marker analysis.

As used herein in the specification and in the claims section that follows the phrases "marker analysis" and "cellular marker analysis" are used interchangeably and include, but are not limited to, chromosomal analysis, such as, for example, fluorescence in-situ hybridization (FISH) or analysis of products directly or indirectly generated from gene expression. For example, analysis of mRNA can be effected by using for example, suitable nucleic acid probes. Analysis of protein expression, can be effected by using, for example, suitable antibody probes, and/or analysis of protein activity can be effected by using suitable reagents depending on the activity to be monitored. In addition, analysis of complex carbohydrates displayed by a cell can be effected by using, for example, antibodies, lectins or various carbohydrate specific chemical reactions.

These two stages are performed by using, in combination, a morphology processing kit (also referred to herein as a pre-processing kit), a scanning unit and a cellular markers processing kit (at times referred to herein also as a post-processing kit).

Rare cell detection is effected, in accordance with the teachings of the present invention, by using, in combination, the morphology processing kit and the scanning unit. The marker analysis is performed via a scanning unit or another imaging unit and the markers processing kit.

In distinction from prior art methods and systems, the system and method of the present invention can be utilized to cross reference between the morphology of individual rare cells and their markers profile, by for example, presenting two images of each cell on the screen, one image that shows the cell morphology and is taken with, for example, bright field illumination and the other image that shows the markers profile of the same

cell. This enables a cytogeneticist to analyze the results with a substantially higher degree of accuracy since when two distinct analysis methods are employed, such as the case with the present invention, the probability for false positive or false negative results is extremely low. In sharp contrast, prior art systems and methods which rely on either morphology or markers analysis are more likely to produce false positive or false negative results.

It will be appreciated, that in addition to the advantages stated above, the system of the present invention enables diagnosis directly from a display screen thus greatly enhancing ease of use.

Pre-processing

In order to better capture morphological features of the rare cells as viewed under bright field illumination and/or fluorescence excitation and as such enhance detection thereof, several pre-processing steps may be undertaken.

Optional non-nucleated cell elimination and rare cell enrichment: When analyzing certain blood samples it may be advantageous to eliminate non-nucleated cells since such cells represent the vast majority of cells in a blood sample and/or since such cells are readily eliminated by simple and highly discriminative procedures of, for example, gradient centrifugation or cell lysis. Non-nucleated cell elimination is also advantageous because it enables screening of more nucleated cells, thus increasing the efficiency of the method and system of the present invention. However, it will be appreciated that this step is not essential to the system and method of the present invention. In any case, the step of elimination as practiced by the present invention is different from enrichment steps practiced in the Background section methods. In sharp contrast to these methods, the elimination step is utilized with great care so as not to eliminate fetal cells. As such, this step does not only enrich fetal blood cells, it also maintains initial fetal cell quantities. The enrichment protocols, on the other hand,

increase the concentration of fetal cells, but at the same time also decrease fetal cell quantities up to 30 % in some cases. Notwithstanding from the above, any of the methods described in the Background section and which are designed for rare cell enrichment can be employed as part of the pre-processing procedures of the present invention.

Bright light staining: Depending on the application, conventional bright light staining procedures are employed by the present invention to uniformly enhance morphological features of the cells. A plurality of bright light stains, also known as histological stains, and procedures can be employed, as is further detailed hereinunder. The specific stain composition can include, in addition to non-specific stains, affinity (antibody/lectin based) bright light stains, which stain protein markers unique to the rare cells such as, for example, fetal hemoglobin. Examples of appropriate histological stains include, but are not limited to 4',6-diamidino-2-phenylindole, Eosin, Fluorescein isothiocyanate, Hoechst 33258, Hoechst 33342, Propidium Iodide, Quinacrine, Fluorescein-phalloidin, Resorufin, hematoxylin, Orange G, Light Green SF, Romanowsky-Giemsa, May-Grunwald, Blue counterstain, ethyl green, Feulgen-naphthol yellow S, Giemsa, Methylene Blue, Methyl Green, pyronin, Naphthol-yellow, Neutral Red, Papanicolaou stain, Red Counterstain C and Sirius Red.

Staining is effected according to preferred embodiments of the present invention in a manner which ensures uniform cell staining of samples from the same individual or from different individuals. This is achieved by modifying common staining protocols to meet the needs of the system and method of the present invention.

In addition, the stain(s) utilized can include specific markers which enable separation of the fetal cells from the maternal cells. A variety of such markers which are known in the art can be utilized by the present invention.

Fluorescence staining: According to the present invention, cell

preparations can also be stained with various fluorescence stains such as but not limited to, Chromomycin A 3, DAPI, Acriflavine-Feulgen reaction, Auramine O-Feulgen reaction, Ethidium Bromide, Propidium iodide, high affinity DNA fluorophores, Green Fluorescent Protein fused to DNA binding protein, ACMA, Quinacrine and Acridine Orange, Feulgen reagent, Gallocyanin chrom-alum, Gallocyanin chrom-alum and naphthol yellow S, Methyl green-pyronin Y and Thionin-Feulgen reagent.

Optionally, such fluorescence staining can be utilized in addition to the bright light staining described above. Such co-staining allows the inspection of the sample both under bright light and under fluorescence excitation without additional sample preparations and thus may present a more accurate method to identify specific cells.

Physical preparation of the sample

It will be appreciated that since the present invention is targeted at the detection of rare cell types, sample preparation and sample presentation must be carefully executed in order to enhance image capturing beyond that effected by prior art systems.

Thus, according to an aspect of the present invention there is provided a novel sample carrier, which is referred to herein as carrier 10.

As is specifically shown in Figure 2, carrier **10** includes the following design features:

(i) A large surface area which is substantially larger than that of typical microscope slides. This feature ensures that relatively large amounts of sample material can be carried by a single carrier **10**. Carrier 10 according to the present invention is of a surface area which is preferably about 60 x 60 mm, more preferably about 70 x 70 mm, most preferably about 75 x 75 mm. As used herein the term about refers to ± 10 %. In addition, and in contrast to prior art sample slides, carrier **10** includes between 60-120 sample wells (one of which is indicated by **12**) as opposed

to a single or several sample wells present in prior art slides. It will be appreciated that carrier **10** described herein is still small enough to be placed in a standard swinging bucket centrifuge.

(ii) Carrier **10** can also include feducial markers **14** provided, for example, at the four corners of the carrier, which can be used to orient the sample on the carrier. Markers **14** preferably feature a cross hair configuration.

(iii) Carrier **10** of the present invention enables effective sample presentation by, for example, utilizing the cyto-centrifuge principle. For example, and as specifically shown in Figure 3, a dedicated dispenser **16** which includes a container **18** provided with a plurality of holes **20** can be placed on top of carrier **10** such that each hole of the dispenser is oriented with a well **12** of carrier **10**. Container **18** can include a single chamber **22** communicating with each hole **20**, or a plurality of chambers **22** in which case each chamber **22** is provided with a dedicated hole or holes **20**. In any case, when in use, a 25-100 microliter, preferably about 50 microliter sample of blood is placed in each chamber **22** of container **18** following which, carrier **10** and dispenser **16** are placed in a centrifuge for a few minutes. As a result, each well **12** is uniformly coated with a layer of blood which typically includes a uniform distribution of a portion of the cell types found in the analyzed sample and, most importantly, is a single cell thick. The uniformity and thickness of the blood sample simplifies an auto-focus procedure which is preferably employed during scanning, thus greatly simplifying this procedure.

(iv) A portion of wells **12** of carrier **10** can be of a configuration which is dedicated for various marker analysis techniques such as, for example, FISH analysis. Thus, following cell detection in such wells FISH staining can be effected under optimal conditions. It will be appreciated that this feature of the carrier also enables cost effective utilization of various FISH-probes since in this case a FISH probe is only applied to a

fraction of the wells.

It will further be appreciated that dispenser **16** or a dedicated cover slip of similar design can be utilized to deliver FISH probes or any other analysis reagents to specific wells **12**. This ensures appropriate provision of the FISH probes. It will be appreciated that such a cover slip can be used in an automated manner thus greatly simplifying the procedure.

Sample scanning

As is specifically shown in Figure 4, to scan the blood sample provided on carrier **10,** the scanning system of the present invention, which is referred to herein as system **30** includes a stage **32** which is designed for holding carrier **10** and for translating carrier **10** along an X, Y and optionally Z axes. Optionally, system **30** includes an automated loader **34** which serves to load carrier **10** onto stage **32.** Stage **32** is attached to a microscope **36** which is preferably provided with both bright light illumination and fluorescence excitation illumination (e.g., ultraviolet illumination). An imaging device **38,** such as CCD camera which can provide gray scale or preferably color images, is attached to microscope 36 in order to capture images provided thereby which images are transferred to a computer **40,** such as a personal computer (PC) or a personal work station, for display and image processing.

Figure 5 is a self explanatory flow chart diagram describing the various steps **(50-54)** utilized by system **30** in order to process a sample of cells. Each image captured by imaging device **38** is processed and analyzed by computer **40** in order to detect and localize, for example, fetal nucleated red blood cells (NRBC) or cancer cells in the sample. To enable such sample processing, system **30** of the present invention inspects a full sample at an analysis rate of approximately 10^3 - 10^4 cells per second, thus enabling to scan a sample containing 10^6- 10^7 cells in less than 30 minutes.

As is further described hereinunder, and in sharp contrast to prior art

designs, the system of the present invention utilizes a combination of high throughput optical scanning which enables fetal cell detection based on morphological and/or spectral characteristics combined with automated sample preparation and scanning.

Algorithm utilized for identification of rare cells

The cell detection method of the present invention implements a novel algorithm for detecting rare cells, such as fetal NRBCs or cancer cells in for example minimal residual disease within blood samples even in cases where such samples have not been enriched for such cell types.

In general, and as specifically shown in Figure 6, the algorithm of the present invention utilizes three steps.

It will be appreciated that this algorithm can be adapted to each sample processed, so as to account for variations in staining, illumination and background optical noise. This adaptation is performed at the end of the trinarization step which is further described hereinbelow.

Following image capturing, as is indicated by **60**, the algorithm of the present invention employs the following steps, in which in a first step, which is referred to herein as "trinarization" and is indicated by **61**, the various image objects are divided into three categories: cell nuclei, cell cytoplasm and sample background. Two binary images are then created: in the first image picture elements (pixels) of cell nuclei and similar objects are marked with a first binary symbol, say "1", while all other pixels are marked with the other binary symbol, say "0". Accordingly, in the second image, pixels of cell cytoplasm and similar objects are marked with a "1" while all other pixels are marked with a "0".

In a subsequent step of the algorithm of the present invention which is referred to herein as "blob finding" and is indicated by **62**, the binary image data is processed so as to locate blobs according to the processed binary data. In the following step, which is referred to herein as "blob

selection" and is marked by **63**, each object characterized in the previous step is processed using both the binary image data and the original color image captured by imaging device **38**. Cell images are analyzed by extraction of a set of features and the cells are classified accordingly.

Trinarization: It will be appreciated that the step of trinarization must effectively derive data on at least most of the objects found in each image. Since the structure and/or size of the cytoplasm and nuclei of NRBCs and/or cancer cells is unique to such cells, the most efficient manner to derive such data would be to separately inspect the shape and size of these objects.

Thus according to a preferred embodiment of the present invention, the trinarization step first generates an adapted threshold to compensate for variations in staining and other parameters of the sample. Since variance of staining reagents and other preparation reagents does exist, there is no way to ensure uniformity in color and contrast of the cell image. Thus, a specific threshold must be adapted to each specific sample, such that results will not be affected by the sample preparation steps utilized. In order to effect such adaptation, system **30** captures sets of images from a current slide, by splitting the scan area into several areas and captures a representative image of each area. A histogram representing each of these images is generated and analyzed, as is further described below, and an optimal trinarization thresholds is determined therefrom.

Figure 7 describes in detail the generation of the histogram described above. Individual channels of color pixels (red channel **70a**, green channel **70b** and blue channel **70c**) of each captured image are converted into weighted monochromatic values (**71a-c**) and thereafter a weighted average (**72**) is calculated for each color pixel. The weighted average is generated by applying different weights for each color channel in order to compensate for variations in the CCD sensor and in order to enhance features present in the image.

Once monochromatic pixels are obtained, a normalized 256 gray scale (0-255) histogram, as is indicated at **73**, is created. This histogram represents pixels from all of the images, thus reflecting the quality of each area captured from the sample. Based on this histogram, independent threshold values may be selected, as is indicated by **74**, for cytoplasm and nucleus. For each threshold, values below the threshold are converted to "0" (zero), while values above it are converted to "1" (one). In one embodiment, this is executed independently for nucleus and cytoplasm in two identical images. Obviously, selection of these thresholds is of great importance since selection of low threshold can lead to falsely identifying objects as background. Alternatively, if the thresholds are selected too high, many objects will be falsely analyzed by the algorithm, thus increasing the run time and may lead to "false positive" results.

Thus, the purpose of the histogram is to produce two thresholds, in order to separate between the nuclei, the cytoplasms and the background. Thus, an optimal threshold depends mainly on three parameters: (i) a mean intensity of the cells' nuclei and their distribution; (ii) a mean intensity of the cells cytoplasms and their distribution; and (iii) a mean intensity of the background and its distribution.

It will be appreciated that due to image quality variations, the algorithm of the present invention preferably generates the thresholds from the monochromatic images which are normalized images.

According to another preferred embodiment of the present invention, threshold determination is effected as follows:

In a first step the histogram is transformed in order to nullify random noise present in the image. This is performed by replacing each value of the histogram by an average of its neighboring values by using the following equation:

$$bin[I] = average(bin[I-2], bin[I-1], .., bin[I+2])$$

Figures 8-9 illustrate a non-transformed histogram (Figure 8) and a transformed histogram (Figure 9). Following this transformation, threshold determination is effected. In the case of a blood sample, for example, cell nucleus, cell cytoplasm and background values each typically follow a bell-shaped curve distribution. Thus, an optimal cell nuclei threshold is represented by a value which is greater than that of a peak value of the nuclei curve (to the right of the peak) and an optimal cytoplasm threshold is represented by a value which is greater than that of a peak value of the cytoplasm curve. In order to detect a peak value, the algorithm searches the histogram for a sequence of increasing values (left slope of hill), followed by a sequence of decreasing values (right slope of hill). This is effected in sequence for both the nuclei and cytoplasmic curves.

As is specifically shown in Figure 10 the algorithm, preferably sets the nuclei threshold in between the two curves - "nuclei hill" and "cytoplasm hill". Similarly, the algorithm, preferably sets the cytoplasm threshold in between the two curves - "cytoplasm hill" and "background hill".

While reducing the present invention to practice it was uncovered that a typical nuclei threshold receives a value of between 60-90 (of a normalized 0-255 gray scale) while a typical cytoplasm threshold falls between 180-220 (of a normalized 0-255 gray scale).

Thus, prior to further utilization the algorithm of the present invention, a determination is made whether the thresholds lie within preset boundary values, which boundaries are determined from statistical analysis of numerous cell images. In cases where a calculated threshold falls outside of these boundaries, the threshold is set to be a new boundary value.

In addition, the algorithm also calculates a ratio between the number of pixels which were classifies as "nuclei" and those classified as "cytoplasm" and ensures this ration falls within a predetermined dynamic

range. Further, the average size of the nucleus and cytoplasm can be calculated and compared to predetermined ranges. Under predetermined sample distribution conditions absolute parameters such as the number of nuclei and/or cytoplasms, as determined from the total number of pixels associated therewith, may also be determined. All of these parameters can be used to score the quality of analysis. As before, these parameters are updated as the system accumulates data. Erroneous parameters are indicative of erroneous thresholds selection. In such case, the thresholds are replaced by preset thresholds which are preferably those calculated based on the entire or a portion of the image database so far acquired by the system. Thus, the system of the present invention constantly quality controls itself and readjust itself so as to optimize its analysis.

Following verification, a set of adjustment factors are applied and the results are translated into a slide specific vector $g_i(s)$. This vector is used in the step of "blob finding" (described herein) as part of the weighted average calculation. This process utilized by the present invention differs from other prior art adaptive processes. Typically, adaptive process are used to set algorithm parameters. In the present invention, the same algorithm is adapted to compensate for, while not being limited to, variation in the staining, variation in the illumination and other optically derived noise.

As is further shown in Figure 7, once thresholds are determined (74), the system of the present invention can start systematically scanning the sample. Nuclei and cytoplasm binary images are then calculated for each color image captured by imaging device **38**. Since the thresholds were predetermined, image processing is simple and fast. As is indicated at 75, first, the color image is converted into a gray level image using pre-defined weights for each color channel. Second, as is indicated at **76**, the algorithm of the present invention utilizes the two predetermined thresholds in order to convert the gray scale (monochromatic) image into two binary images: the first threshold sets the "nuclei image" while the second threshold sets

the "cytoplasm image".

Blob finding: Blob finding serves according to the present invention to locate all objects of an image and to filter out all the objects which are of an undesired type.

Figure 11 illustrates "blob finding" which initiates, as is indicated at **111** with a connected components algorithm, which separately processes the binary nuclei and cytoplasmic images by labeling each contiguous component with a different label. Once the binary image is labeled, each labeled component is considered a "blob", and a set of features, as indicated at **112**, is extracted for each blob. Since the set of features is extracted from a binary image, it includes features, as indicated at **113**, relating to the shape of the objects. A typical set of features includes, but is not limited to, (i) a size of each blob, which represents the number of pixels therein multiplied by the physical size of each pixel; (ii) a perimeter of each blob, which represents the number of pixels present along the border of each blob; (iii) a roughness of each blob, which represents the ratio between the perimeter and the convex-perimeter of each blob; (iv) a compactness of each blob, which represents the ratio between the size and the diameter of each blob; (v) an elongation of each blob which represents a ratio between the length of the blob and its width; (vi) bounding box dimensions; (vii) minimal diameter; (viii) maximal diameter; (ix) mean diameter; (x) convex perimeter; (xi) minimal intesity of a pixel; (xii) maximal intesity of a pixel; (xiii) mean intesity of all pixels; (xiv) standard deviation of pixel intesity; (xv) sum of squares of pixels intesities; and (xvi) a number of "holes" of each blob which represents the number of discontinuous regions present in each blob.

The processed nuclei and cytoplasmic blobs are matched according to their coordinates and additional features are then extracted by determining: (i) a ratio between each cytoplasm and its respective nucleus; (ii) a level of overlap between each cytoplasm and its respective nucleus

(full/partial); (iii) a minimal distance between the edges of the nucleus and the cytoplasm; and (iv) a maximal distance between the edges of the nucleus and the cytoplasm.

Each of these features may be calculated for any of the following planes: (i) independently for the red, green and blue planes; (ii) independently for hue saturation and intensity (HSI) planes; and (iii) for an edge detection image generated as described herein.

It will be appreciated that when overlap between adjacent cytoplasms occurs, calculation of some of the above features cannot be determined. As such, a non-significant score is assigned to such cases.

Following feature determination, a comparison between the above determined features and a feature database is effected.

This database stores values of features of stored image libraries which were meticulously classified by experts. Thus, the calculation of features extracted from the analyzed image is performed by comparing its value to values stored by the database. As indicated at **114**, each comparison is given a probability score which classifies an object to a class of images characterized by this feature. Discrimination theory techniques are used at this point to determine the scores and weights when a features distribution of an object is known. Alternatively, fuzzy logic techniques are utilized in determining scores and weights of an object of unknown distribution.

When the scores of each feature are calculated, as indicated at 115, a weighted sum is assigned thereto and a final score is determined for each object. The weights are distributed such that larger value are assigned to features that are very important hence increasing their score. It will be appreciated in this case that no single parameter dominates the classification but rather it is a combination of all the parameters and their values which influences the score.

If an object receives a total score which is above a pre-defined

threshold, it is processed by a subsequent "blob selection" step which is further described hereinbelow. In such a case, the set of features already calculated for this object is stored by a computer memory.

Mathematically the weighted sum can be expressed by the following equation:

$$F = S_i\, g_i(s) * a_i * f_i$$

where S_i is a summation of the above described parameters $g_i(s)$ is the adaptation vector described hereinabove, a_i is the weight of the current feature and f_i is the feature itself.

Blob selection: As is shown in Figure 12, at the blob selection step, the algorithm of the present invention inspects each object and decides if it is of the desired type. This is processed, as indicated at **121**, from the following inputs: (i) two binary images (1 bit per pixel) are generated by cropping a larger binary image such that the cropped images place the object in their centers; (ii) a color image is cropped from the larger color image captured in the previous step such that the cropped image places the object in its center; (iii) a set of features calculated for this object and which are used as negative selection parameters therein, are utilized by this step as positive selection parameters in order to identify objects of interest.

The blob selection step is utilized to correctly identify cell types. In general, this recognition algorithm is based on feature extraction followed by feature evaluation. Although extraction of some of these features is CPU intensive, this step is performed only on a subset of the objects and as such, its CPU utilization can be relatively minor providing sophisticated algorithms are utilized.

Nucleus boundary detection: Accurate nucleus boundary detection is effected by analyzing the binary nuclei image generated by previous steps. This detection is achieved according to the present invention by thresholding the image. As is shown in Figure 13, which is self

explanatory, an iterative algorithm is utilized to expand the nucleus image thus accurately detecting nucleus boundaries. Thus, in a first step, as indicated at **131**, all nucleus boundary pixels are added to a boundary set. In a second step, as indicated at **132**, an initial threshold value is set. In a third step, as indicated at **133**, a first pixel of the boundary set is selected at random. In a fourth step, as indicated at **134**, a first neighboring pixel of the first pixel is selected outside the nucleus. At this stage, as indicated by 135, a gray level comparison between these pixels is effected. If similar, as indicated by **136**, the neighboring pixel is compared to the threshold. If it falls within the threshold, as indicated by **137**, the neighboring pixel is added to the boundary set. If the gray level comparison between these pixels results in non-similarity, than, as indicated at **138**, if not all of the neighboring pixels have so far been examined, another neighboring pixel, as indicated at **139**, is selected for a similar analysis. Similarly, if the neighboring pixel has a gray level which falls outside the threshold, than, it is rejected from the boundary set of pixels and another neighboring pixel, as indicated at **139**, is selected for a similar analysis. As indicated at 140, when all of the neighboring pixels have been analyzed as described above, the last neighboring pixel is removed from the boundary set. Once all of the pixels are removed from the boundary set, as indicated by **141**, a new threshold value is set, as indicated by **142**, and when the above process is executed for all threshold values in a predefined threshold range, as indicated at **143**, and for all the pixels in the boundary set, as indicated at **145**, nucleus boundary determination is completed, as indicated at 144, whereas all the pixels of the boundary set are either added or rejected from the nucleus. This procedure thus results in nucleus segmentation as is identified at **124** in Figure 12.

Cytoplasm segmentation: Since it is common for two or more cytoplasmic areas to overlap, an external ring of a pre-defined width (e.g., 2 or 3 pixels) around the nucleus is considered by the algorithm of the present

invention as the cytoplasm. Since it is assumed that this ring is situated inside the actual cytoplasm it is considered a correct representation of the color features of the cytoplasm. Using this procedure, the cytoplasm is segmented as is identified at **125** in Figure 12.

Once the nucleus and the cytoplasm are segmented, the algorithm of the present invention extracts color features from the pixels of the nucleus and the cytoplasm as is further described below.

Nuclear and cytoplasmic color features: As is further shown in Figure 12 and indicated there at **122**, the color data of both the nucleus and the cytoplasm is extracted from captured and segmented color images. The parameters extracted can include, but are not limited to, (i) an average intensity for each of the color channels (e.g., red, green and blue); (ii) a standard deviation in the intensity of each color plane; and (iii) an average hue and saturation which are obtained by an RGB to HSI transformation of the color image.

In addition to the above, as is indicated at **123**, several additional features of the nucleus and the cytoplasm can also be extracted from the segmented color image. For example, a contrast of the nucleus, which is defined as the contrast between the nucleus edge and its contour can be determined. Such determination can be effected by preparing a binary image of the contour of the nucleus, and employing an edge detection algorithm, for example, the Sobel algorithm, which can be applied to a color image. The edge and contour images can then be superimposed and a binary contrast score generated, such that an average contrast is readily determined.

Other features can be determined from a difference between an average hue of the nucleus and cytoplasm, shape features of the nucleus, which are determined as described hereinabove for "Blob finding". It will be appreciated that the "nucleus segmentation" step may alter the shape features determined in the "Blob finding" step. In such a case, these

features are recalculated.

Classification: The above described analysis steps employed by the algorithm of the present invention enable the extraction of numerous features which form an N dimensional vector $f_i(o)$ for each object in an image, wherein i indexes the feature type. According to the teachings of the present invention this feature vector is used to classify a cell type.

As is shown in figure 14, the algorithm of the present invention analyzes the features vector and calculates a score for a current cell type. These features can be classified into three categories:

(a) As indicated at **140**, *a mandatory feature* classifies a cell according to a pre-defined feature score (**141**), otherwise, classification is rejected (**142**), regardless of other positive classifying features.

(b) As indicated at **143**, *a winner feature* or *a sufficient feature* classifies a cell regardless of the outcome for other features.

(c) As indicated at 144, *a standard feature* contributes to the final score of a cell, but cannot define the final classification by itself.

Thus, the features of the mandatory set are correlated with a normalized threshold. In other words, for each feature there is a check whether the product of the slide normalization factor ($g_i(s)$) and the weighted value of the feature (a_i*f_i) is above a preset value. The thresholds are determined based on a statistical analysis previously done on an image library.

If the conditions dictated by all of the mandatory features are met, then the analysis of the winner features is performed. On the other hand, if one or more of the mandatory features is below a minimal value the cell is classified as one of no interest.

The winner analysis is performed and compared to a predetermined minimal value, if the sum of $W_S = S\, g_i(s) * a_i f_i$ is above the minimal value the cell is positively classified, if not, then the next processing step is effected.

The standard features analysis is calculated for the standard features S_S and added to a winners' sum (W_S), identified at **145**. The resulting value is compared to a preset value. If it is above the preset value, then, as indicated by **146**, the cell is positively classified and vice versa.

It will be appreciated that the various steps described hereinabove can only be effected via the algorithm of the present invention. Although prior art image processing algorithms can be used to differentiate between cell types, such algorithms typically differentiate between images of different cell types by treating each cell as a single processed object and not as a combination of processed objects as performed by the algorithm of the present invention.

For example, WO 97/20198 and U.S. Pat. No. 5,764,792 describe methods for identifying rare cells such as, cancer cells, in a sample according to their affinity to acidic and basic dyes. These method employ algorithms which detect such cells and thereafter, based on whole cell morphologic analysis, such as shape and size, classify the cells thus detected. Thus, not only the initial screening is based on color image analysis, the morphological study fails to segment the cells and separately address the nucleus and cytoplasm.

Although the algorithms employed by prior art methods are capable of distinguishing some cell types from others, their processing mode limits such algorithms to the identification of morphologically distinct cell types. In sharp contrast, since the algorithm of the present invention enables a more accurate cell characterization process which relays on characterizing a plurality of objects which are associated with each cell analyzed, and according to a plurality of features, such an algorithm can be utilized to distinguish between morphologically similar cells of a different type or between, for example, singular multinucleated cells and clumps of mononucleated cells.

Specific cell type recognition

Specific cell recognition is used according to preferred embodiments of the present invention to achieve one or both of the following goals: (i) cell recognition; and (ii) quality control examination for the efficiency of the above described algorithm in rejecting cells of no interest..

As is shown in Figure 12, indicated by **126 and 127,** the system and method of the present invention are adapted at recognizing specific cell types, such as, but not limited to, neutrophills and lymphocytes

Neutrofills recognition:

In order to detect neutrofills, the algorithm of the present invention searches for a fragmented nucleus. A fragmented nucleus image includes two or more separate nuclei, which share a single cytoplasm. To accurately detect neutrophilles, the algorithm of the present invention must be able to distinguish between adjacent cells, and a neutrophill. This is achieved by searching for additional objects which share a single cytoplasm with the analyzed object.

First, the algorithm searches for additional dark objects positioned a pre-defined distance away from the object analyzed. If such an object is detected, the algorithm checks the colors in the bounding convex contour, of the two objects. If the contour includes background (white) pixels, the two objects are considered separate nuclei (as shown in Figure 15a). However, if background (white) pixels are not detected, the two objects are considered two fragments of the same nucleus (as shown in Figure 15b).

It will be appreciated that additional measures can be utilized to distinguish between adjacent cells and a neutrophill. These measures include, but not limited to, (i) cytoplasm curvature, which is present between the two dark objects in the case of adjacent nuclei and is absent in the case of a neutrophill; (ii) a contrast variation along connecting lines which analyzes the gray level along straight lines connecting the two dark object; in the case of a neutrophill, smaller variation of gray level along

such lines exists as is compared to the case of adjacent cells.

Lymphocyte recognition:

Lymphocytes are detected according to the present invention by detecting two defining characteristics: a small cytoplasm and a relatively large nucleus.

Thus, for each object type the algorithm of the present invention evaluates these features to determine if a cell analyzed is a lymphocyte.

When, for example, NRBCs or cancer cells are to be identified among a mixed population of other blood cells, following identification thereof, quality control may be applied, for example, by attempting to recognize neutrophills and/or lymphocytes among the cells identified.

NRBCs recognition:

The following provides description of a presently best mode of practicing the present invention in identification of NRBCs using the image processing algorithm herein described. Table 2 below provides presently preferred values for the features of the algorithm utilized by the present invention.

Table 2

Feature	Value
Nucleus size (pixels)	60-170
Nucleus compactness (pixels)	< 1,800
Elongation (pixels)	< 1,500
Average nucleus saturation (intensity *)	> 30
Average nucleus luminance (intensity *)	> 30
Averaged nucleus blue intensity (intensity *)	> 80
Averaged nucleus red intensity (intensity *)	> Averaged nucleus blue intensity
Averaged nucleus green intensity (intensity *)	< Averaged nucleus blue intensity - 10
Average cytoplasm luminance (intensity *)	> 190

* in 0-255 normalized scale

Using these values, the system of the present invention had the following performance: of 200,000 maternal derived white blood cells, containing a total of 25 NRBCs as was determined manually by a qualified

cytogenetecist, a total of 30 objects were identified as NRBCs. Of these objects 20 were indeed NRBCs while the remaining 10 objects were either lymphocytes or non-cellular objects.

Cellular markers analysis

The system and method of the present invention preferably employs additional cellular markers analysis steps which are utilized to further validate the results obtained from image processing as described hereinabove.

To effect such an analysis, system **30** of the present invention can be utilized to further characterize cells which were identified as fetal or cancer cells according to the image processing algorithm of the present invention via various chemical, biochemical and/or molecular techniques (herein cellular marker analysis).

Such chemical, biochemical and/or molecular techniques can be utilized by either applying the appropriate reagents to all the cells in advance before effecting image processing or by applying the reagents following image processing and fetal cell determination.

In the former case, system **30** identifies the location of the detected fetal or cancer cells and specific images of the rare cells under, for example, fluorescence excitation illumination are obtained for analysis.

In the latter case, system **30** simply identifies the location of the fetal cells, provides the necessary reagents and conditions in a localized fashion and specific images of the rare cells under, for example, fluorescence excitation illumination are obtained for analysis.

It will be appreciated that this feature of the present invention is distinct from prior art methods in which cellular marker analysis is typically performed on the entire slide area.

In addition, system **30** of the present invention can further include a manual or an automatic (robotic) device which can be utilized to collect the

detected fetal cells. This may be done by means of a mechanical micro manipulator or a laser dissection device. The cells that are picked up in this manner can be analyzed in any one of a plurality of molecular methods, such as, for example, PCR amplification followed by gel electrophoresis and/or any one of a plurality of methods for mutation detection, such as, for example, nucleic acid sequencing.

Chromosomal analysis:

Chromosomal aberrations are characteristic of certain diseases. For example, chromosome 21 trisomy is characteristic of down syndrome. Certain chromosomal rearrangements, such as the Philadelphia chromosome in chronic lymphatic leukemia (CLL) and the a specific chromosomal aberration in Burkitt's lymphoma are also well documented. These chromosomal aberrations, as well as many other, are readily identifiable using known techniques (e.g., FISH) and/or instrumentation (e.g., spectral imaging systems). To this end, see, for example, U.S. Pat. Nos. 5,719,024; 5,798,262; 5,871,932; 5,906,919; 5,912,165; and 5,936,731 assigned to Applied Spectral Imaging Ltd., Israel, which are incorporated herein by reference.

mRNA analysis:

In addition to chromosomal analysis, the present invention can also utilize methods for detecting or quantitating specific mRNA species. Such methods can be effected by utilizing for example, fluorescently tagged probes. Thus, using such analysis NRBCs can be detected by probes specific for fetal hemoglobin mRNA species.

Analysis of cell associated proteins or carbohydrates:

Certain proteins, glycoproteins and/or surface carbohydrates are characteristic of some rare cell types. For example, NRBCs include fetal hemoglobin species. Certain cancer cells present tumor associated or tumor specific antigens on their surface. Such antigens may be portions of proteins, glycoproteins and/or surface carbohydrates which are recognizable

using antigen specific affinity stains, typically including an antibody directed against such antigens, examples of which are given hereinabove.

Synergizing between morphological identification and cellular marker analysis

It will be appreciated that in order to improve an analysis inherently susceptible to a high level of false positive results, such as in the case of rare cell identification, one may use independent identification techniques and thereafter cross their results, so as to substantially reduce the false positive results.

In view of the results in NRBCs identification described above, it may prove advantageous to combine an independent NRBCs identification procedure, so as to reduce the degree of false positive results (33 % in the example given).

For NRBCs identification, such an independent identification technique may, for example, include, fetal hemoglobin, fetal hemoglobin mRNA, Y chromosome detection. For other rare cells, such as, for example, cancer cells, identifying a variety of cellular markers, as is further detailed hereinabove, may alternatively be employed.

It will be appreciated that three possibilities exist to stain a single sample with both a histological stain and an affinity stain: (i) co-staining; (ii) successive staining wherein the histological stain precedes the affinity stain; and (iii) successive staining wherein the histological stain proceeds the affinity stain. It will further be appreciated that choosing an option of these three options very much depends on the specific nature of the stains employed. In any case, even if co-staining is impossible, successive staining is applicable as long as the location of cells of interest is registered. One of ordinary skills in the art would know how the select among these staining options for a given pair of stains.

Thus, the image. analysis algorithm according to the present

invention can employ any one of the known prior art algorithms which are capable of pointing out uniquely monochromatically colored cells.

Thus, in order to improve the detection of rare cells in a mixed population of cells, according to this preferred mode of the present invention, the identification results obtained by the image processing algorithm utilized by the system and method of the present invention are crossed with independent results obtained by a known prior art algorithm which is capable of pointing out uniquely colored cells (e.g., as described in WO 97/20198 and U.S. Pat. No. 5,764,792, both of which are incorporated by reference as if fully set forth herein), to thereby reduce the presence of false positives.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications cited herein are incorporated by reference in their entirety. Citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

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WHAT IS CLAIMED IS:

1. A sample carrier for mounting over a microscope stage and for placement in a swinging bucket centrifuge, the sample carrier comprising a surface area greater than about 3,500 squared millimeters, said surface area being formed with at least 50 sample wells.

2. The sample carrier of claim 1, further comprising at least two feducial markers.

3. The sample carrier of claim 2, wherein said feducial markers feature a cross hair configuration.

4. A dispenser comprising a container being formed with a plurality of holes, the dispenser being adapted to match to a sample carrier including a surface area greater than about 3,500 squared millimeters, said surface area being formed with at least 50 sample wells, such that when the dispenser is mounted over said carrier, said plurality of holes of said container face said plurality of wells of said carrier, so as to facilitate dispensing of a sample through said holes into said wells.

5. The dispenser of claim 4, wherein said container includes partitions dividing said container into chambers, whereas each of said chambers is in fluid communication with at least one of said holes.

6. The dispenser of claim 4, wherein said holes are positioned at distal ends of protrusions protruding externally to said container.

7. A sample preparation assembly comprising:

(a) a dispenser including a container being formed with a plurality of holes; and

(b) a sample carrier including a surface area greater than about 3,500 squared millimeters, said surface area being formed with at least 50 sample wells, such that when said dispenser is mounted over said carrier, said plurality of holes of said container face said plurality of wells of said carrier, so as to facilitate dispensing of a sample through said holes into said wells.

8. A method of dispensing a sample, the method comprising the steps of:

(a) providing a preparation assembly including:

(i) a dispenser including a container being formed with a plurality of holes; and

(ii) a sample carrier including a surface area greater than about 3,500 squared millimeters, said surface area being formed with at least 50 sample wells;

(b) mounting said dispenser over said carrier such that said plurality of holes of said container face said plurality of wells of said carrier, so as to facilitate dispensing of the sample through said holes into said wells; and

(c) dispensing said sample through said holes into said wells.

9. The method of claim 8, wherein said step of dispensing said sample through said holes into said wells is effected under centrifugal force.

10. The method of claim 9, wherein provisions are taken to ensure that each well includes a monolayer of cells derived from the sample.

11. A method of analyzing a sample, the method comprising the steps of dispensing the sample by:

(a) providing a preparation assembly including:

(i) a dispenser including a container being formed with a plurality of holes; and

(ii) a sample carrier including a surface area greater than about 3,500 squared millimeters, said surface area being formed with at least 50 sample wells;

(b) mounting said dispenser over said carrier such that said plurality of holes of said container face said plurality of wells of said carrier, so as to facilitate dispensing of the sample through said holes into said wells; and

(c) dispensing said sample through said holes into said wells; and analyzing a content of at least one of said wells.

12. A method of detecting and analyzing rare cells in a mixed population of cells carried by a carrier, the method comprising the steps of:

(a) using an automatic or semi-automatic optical scanning system for morphologically identifying the rare cells on the carrier, to thereby obtain identified rare cells of known locations; and

(b) chemically, biochemically and/or genetically analyzing said identified rare cells, thereby validating a result of said step of morphologically identifying the rare cells on the carrier.

13. The method of claim 12, further comprising the step of co-displaying a morphological image and an image presenting a chemical, biochemical and/or genetic analysis of said identified rare cells.

14. A system for identifying rare cells in a mixed population of cells carried by a carrier, the system comprising a scanning unit including an optical magnification device optically communicating with an imaging device and a processing device for analyzing images being acquired by said imaging device, said processing device being for executing an image processing algorithm, said image processing algorithm being for segmenting an image acquired by said imaging device into a first group of pixels associated with cell nuclei, a second group of pixels associated with cell cytoplasms and a third group of pixels associated with background to thereby enable identification of the rare cells.

15. The system of claim 14, wherein said first, second and third groups of pixels are segmented according to at least two thresholds calculated by said image processing algorithm.

16. The system of claim 15, wherein said at least two thresholds are calculated by:

(i) transforming a color image of said cells into a monochromatic image in which each color channel of each pixel is weighted according to its respective intensity and a monochromatic weighted average intensity is given to each of said pixels;

(ii) generating a histogram representing a distribution of monochromatic weighted average intensity distribution among said pixels; and

(iii) using said histogram, calculating said at least two thresholds.

17. The system of claim 15, wherein segmenting an image acquired by said imaging device into a first group of pixels associated with cell nuclei, a second group of pixels associated with cell cytoplasms and a third group of pixels associated with background is effected by generating

at least a nuclei binary image and a cytoplasm binary image.

18. The system of claim 17, wherein said image processing algorithm is further for blob classification, whereas blob classification is effected by, first, identifying connected components in said binary images, thereby identifying blobs in each of said binary images, and thereafter scoring features extracted from said blobs to thereby classify said blobs.

19. The system of claim 18, wherein said features are selected from the group consisting of (i) a size of each of said blobs; (ii) a perimeter of each of said blobs; (iii) a roughness each of said blobs; (iv) a compactness of each of said blobs; (v) an elongation of each of said blobs; (vi) bounding box dimensions; (vii) minimal diameter; (viii) maximal diameter; (ix) mean diameter; (x) convex perimeter; (xi) minimal intensity of a pixel; (xii) maximal intensity of a pixel; (xiii) mean intensity of pixels; (xiv) standard deviation of pixel intensity; (xv) sum of squares of pixels intensities; and (xvi) a number of holes of each of said blobs.

20. The system of claim 14, wherein said first group of pixels associated with said cell nuclei is redefined using a blob finding and classification algorithm and a boundary detection algorithm. .

21. The system of claim 20, wherein said second group of pixels associated with said cell cytoplasms is redefined using a result of said boundary detection algorithm.

22. The system of claim 18, wherein said image processing algorithm is further for matching blobs derived from each of said binary images and thereafter scoring features extracted from matched blobs to thereby classify said blobs.

23. The system of claim 22, wherein said features are selected from the group consisting of (i) a size ratio between blobs of matched blobs; (ii) a level of overlap between blobs of matched blobs; (iii) a minimal distance between edges of blobs of matched blobs; and (iv) a maximal distance between edges of blobs of matched blobs.

24. The system of claim 22, wherein said image processing algorithm is further for extracting color features of pixels associated with said matched blobs.

25. The system of claim 22, wherein said image processing algorithm is further for identifying the rare cells according to mandatory features and sufficient features.

26. A method of identifying rare cells in a mixed population of cells carried by a carrier, the method comprising the steps of:

(a) providing a scanning unit including:

 (i) an optical magnification device optically communicating with an imaging device; and

 (ii) a processing device being for analyzing images acquired by said imaging device, said processing device being for executing an image processing algorithm,

(b) acquiring, via said imaging device, an image of said mixed population of cells; and

(c) utilizing said image processing algorithm for segmenting said image of said mixed population of cells acquired by said imaging device into a first group of pixels associated with cell nuclei, a second group of pixels associated with cell cytoplasms aa third group of pixels associated with

background to thereby enable identification of the rare cells.

27. The method of claim 26, wherein said step of segmenting said image of said mixed population of cells into said first, second and third groups of pixels is effected according to at least two thresholds calculated by said image processing algorithm.

28. The method of claim 27, wherein said at least two thresholds are calculated by:

(i) transforming a color image of said cells into a monochromatic image in which each color channel of each pixel is weighted according to its respective intensity and a monochromatic weighted average intensity is given to each of said pixels;

(ii) generating a histogram representing a distribution of monochromatic weighted average intensity distribution among said pixels; and

(iii) using said histogram, calculating said at least two thresholds.

29. The method of claim 26, wherein said step of segmenting said image of said mixed population of cells into said first, second and third groups of pixels is effected by generating at least a nuclei binary image and a cytoplasm binary image.

30. The method of claim 29, wherein said step of segmenting said image of said mixed population of cells into said first, second and third groups of pixels is effected by blob classification, whereas blob classification is effected by, first, identifying connected components in said binary images, thereby identifying blobs in each of said binary images, and thereafter scoring features extracted from said blobs to thereby classify said blobs.

31. The method of claim 30, wherein said features are selected from the group consisting of (i) a size of each of said blobs; (ii) a perimeter of each of said blobs; (iii) a roughness each of said blobs; (iv) a compactness of each of said blobs; (v) an elongation of each of said blobs; (vi) bounding box dimensions; (vii) minimal diameter; (viii) maximal diameter; (ix) mean diameter; (x) convex perimeter; (xi) minimal intensity of a pixel; (xii) maximal intensity of a pixel; (xiii) mean intensity of pixels; (xiv) standard deviation of pixel intensity; (xv) sum of squares of pixels intensities; and (xvi) a number of holes of each of said blobs.

32. The method of claim 26, wherein said first group of pixels associated with said cell nuclei is redefined using a blob finding and classification algorithm and a boundary detection algorithm.

33. The method of claim 32, wherein said second group of pixels associated with said cell cytoplasms is redefined using a result of said boundary detection algorithm.

34. The method of claim 29, wherein said image processing algorithm is further for matching blobs derived from each of said binary images and thereafter scoring features extracted from matched blobs to thereby classify said blobs.

35. The method of claim 34, wherein said features are selected from the group consisting of (i) a size ratio between blobs of matched blobs; (ii) a level of overlap between blobs of matched blobs; (iii) a minimal distance between edges of blobs of matched blobs; and (iv) a maximal distance between edges of blobs of matched blobs.

36. The method of claim 34, wherein said image processing algorithm further serves for extracting color features of pixels associated with said matched blobs.

37. The method of claim 26, wherein said rare cells are identified according to mandatory features and sufficient features.

38. A method of reducing false positive results while identifying rare cells in a mixed population of cells, the method comprising the steps of:

(a) employing a first algorithm for identifying the rare cells, said first algorithm identifying the rare cells according to morphological features thereof; and

(b) independently employing a second algorithm for identifying the rare cells, said second algorithm identifying the rare cells according to cellular markers characterizing the rare cells;

(c) identifying a specific cell of the mixed population of cells as a rare cell only if both said first algorithm and said second algorithm identified said specific cell as rare.

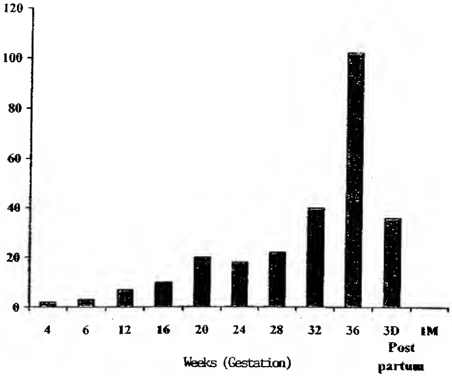
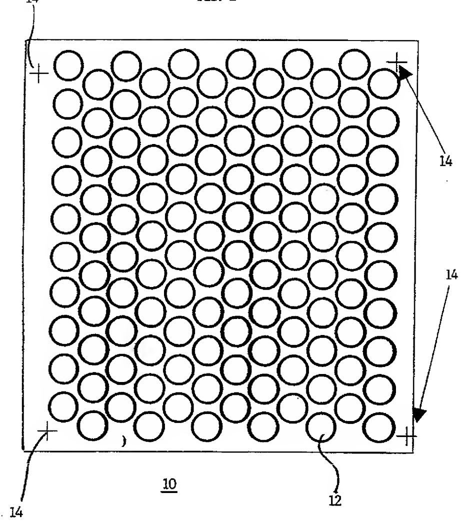

120
100
80
60
40
20
0
4
6
12
16
20
24
28
32
36
3D
1M
Post
partum
Weeks (Gestation)

FIG. 1

FIG. 2



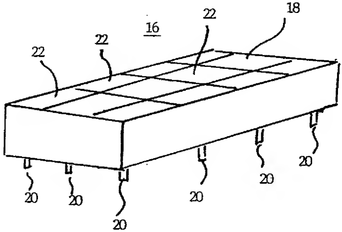


FIG. 3

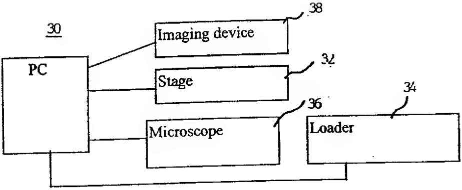

30
PC
Imaging device
38
Stage
32
Microscope
36
Loader
34

FIG. 4

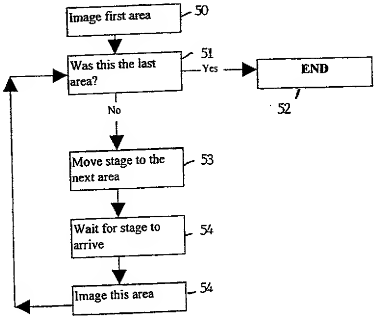


Image first area
50
Was this the last area?
51
Yes
END
52
No
Move stage to the next area
53
Wait for stage to arrive
54
Image this area
54

FIG. 5

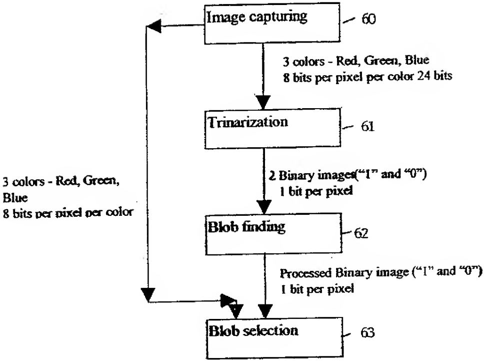
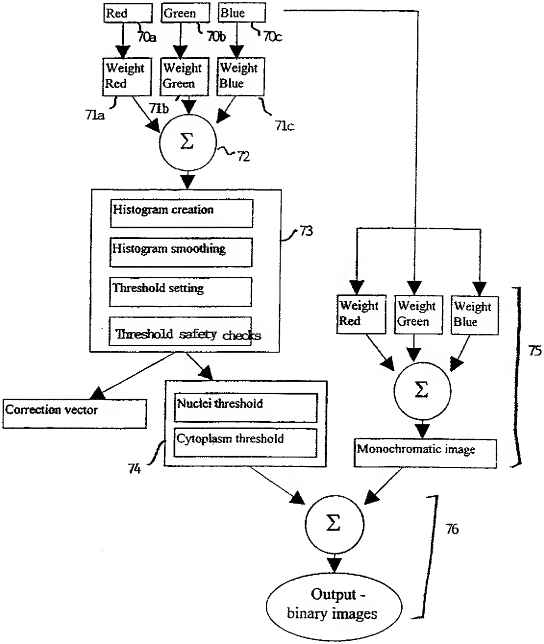


Image capturing
60
3 colors - Red, Green, Blue
8 bits per pixel per color 24 bits
Trinarization
61
2 Binary images("1" and "0")
1 bit per pixel
Blob finding
62
Processed Binary image ("1" and "0")
1 bit per pixel
Blob selection
63
3 colors - Red, Green, Blue
8 bits per pixel per color

FIG. 6



Red
Green
Blue
70a
70b
70c
Weight Red
Weight Green
Weight Blue
71a
71b
71c
Σ
72
Histogram creation
Histogram smoothing
Threshold setting
Threshold safety checks
73
Correction vector
Nuclei threshold
Cytoplasm threshold
74
Weight Red
Weight Green
Weight Blue
Σ
Monochromatic image
75
Σ
76
Output - binary images

FIG. 7

FIG. 8

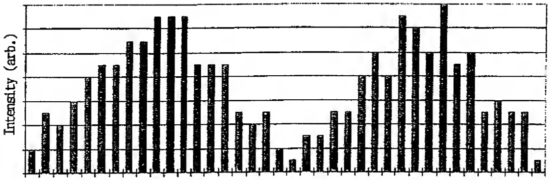
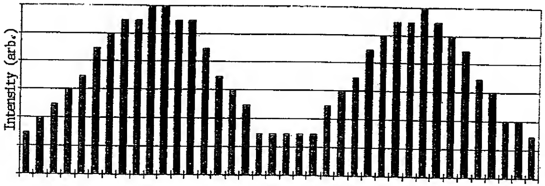


FIG. 9



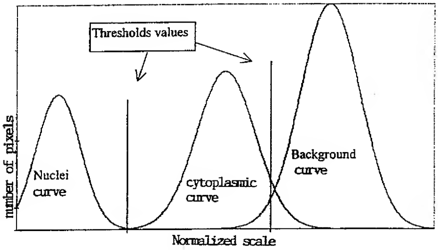

Thresholds values
number of pixels
Nuclei curve
cytoplasmic curve
Background curve
Normalized scale

FIG. 10

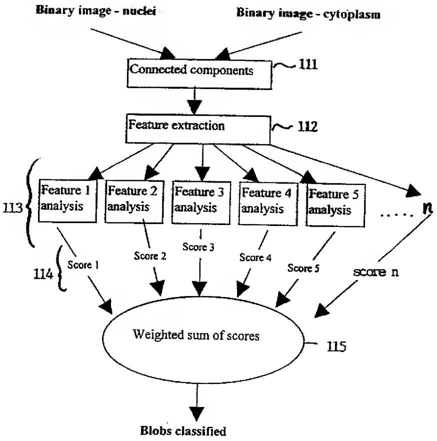

Binary image - nuclei
Binary image - cytoplasm
Connected components
111
Feature extraction
112
113
Feature 1 analysis
Feature 2 analysis
Feature 3 analysis
Feature 4 analysis
Feature 5 analysis
..... n
114
Score 1
Score 2
Score 3
Score 4
Score 5
score n
Weighted sum of scores
115
Blobs classified

FIG. 11

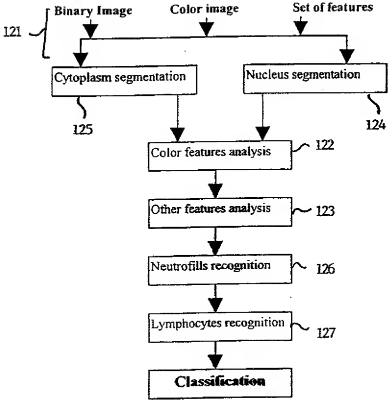

Binary Image
Color image
Set of features
121
Cytoplasm segmentation
Nucleus segmentation
125
124
Color features analysis
122
Other features analysis
123
Neutrofills recognition
126
Lymphocytes recognition
127
Classification

FIG. 12

9/10

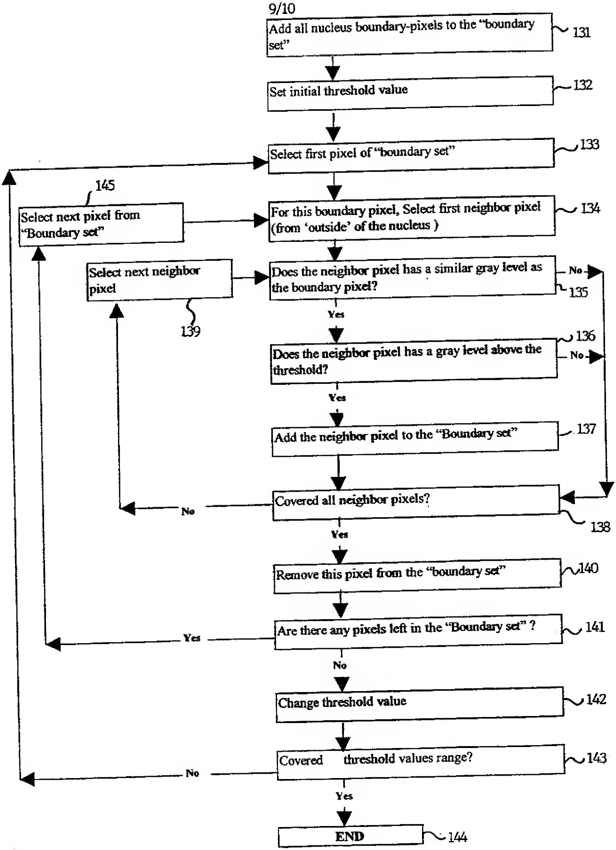

Add all nucleus boundary-pixels to the "boundary set"
131
Set initial threshold value
132
Select first pixel of "boundary set"
133
145
Select next pixel from "Boundary set"
For this boundary pixel, Select first neighbor pixel (from 'outside' of the nucleus)
134
Select next neighbor pixel
139
Does the neighbor pixel has a similar gray level as the boundary pixel?
No
135
Yes
Does the neighbor pixel has a gray level above the threshold?
No
136
Yes
Add the neighbor pixel to the "Boundary set"
137
Covered all neighbor pixels?
No
138
Yes
Remove this pixel from the "boundary set"
140
Are there any pixels left in the "Boundary set" ?
Yes
141
No
Change threshold value
142
Covered threshold values range?
No
143
Yes
END
144

FIG. 13

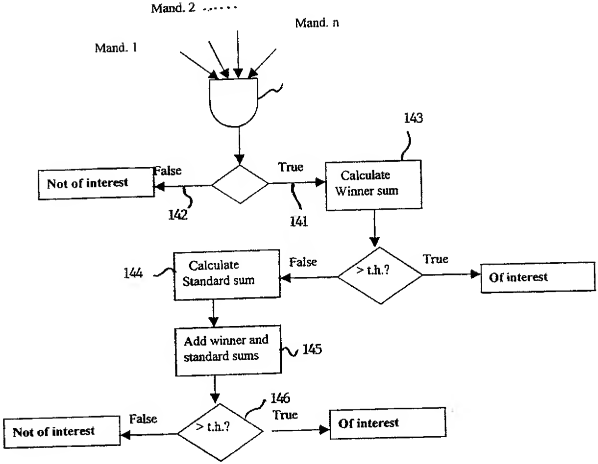
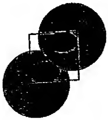


FIG. 14

FIG. 15a

FIG. 15b



INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL00/00101

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :G01N 21/01, 21/00
US CL :356/244, 344; 435/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 356/244, 344; 435/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EAST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,447,842 A (SIMONS) 05 September1995, (05/09/95) col.4, line 10-col.23, line 45.	1-38
Y	US 4,322,298 A (PERSIDSKY) 30 March 1982, (30/03/82) col.8, line 19- col.14, line 34.	1-38
Y	US 5,736,410 A (ZARLING et al) 07 April 1998, (07/04/98) entire document.	1-38

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 MAY 2000	21 JUN 2000

Name and mailing address of the ISA/US	Authorized officer
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